A novel multidrug efflux transporter gene of the major facilitator superfamily from *Candida albicans* (*FLU1*) conferring resistance to fluconazole

David Calabrese, Jacques Bille and Dominique Sanglard

Author for correspondence: Dominique Sanglard. Tel: +41 21 314 40 83. Fax: +41 21 314 40 60. e-mail: Dominique.Sanglard@chuv.hospvd.ch

Institut de Microbiologie, Centre Hospitalier Universitaire Vaudois (CHUV), Rue de Bugnon, CH-1011 Lausanne, Switzerland

Azole resistance in *Candida albicans* can be mediated by several resistance mechanisms. Among these, alterations of the azole target enzyme and the overexpression of multidrug efflux transporter genes are the most frequent. To identify additional putative azole resistance genes in *C. albicans*, a genomic library from this organism was screened for complementation of fluconazole hypersusceptibility in *Saccharomyces cerevisiae* YKKB-13 lacking the ABC (ATP-binding cassette) transporter gene *PDR5*. Among the *C. albicans* genes obtained, a new gene was isolated and named *FLU1* (fluconazole resistance). The deduced amino acid sequence of *FLU1* showed similarity to *CaMDR1* (formerly *BEN*), a member of the major facilitator superfamily of multidrug efflux transporters. The expression of *FLU1* in YKKB-13 mediated not only resistance to fluconazole but also to cycloheximide among the different drugs tested. The disruption of *FLU1* in *C. albicans* had only a slight effect on fluconazole susceptibility; however, it resulted in hypersusceptibility to mycophenolic acid, thus suggesting that this compound could be a substrate for the protein encoded by *FLU1*. Disruption of *FLU1* in a background of *C. albicans* mutants with deletions in several multidrug efflux transporter genes, including *CDR1*, *CDR2* and *CaMDR1*, resulted in enhanced susceptibility to several azole derivatives. *FLU1* expression did not vary significantly between several pairs ofazole-susceptible and azole-resistant *C. albicans* clinical isolates. Therefore, *FLU1* seems not to be required for the development ofazole resistance in clinical isolates.

Keywords: multidrug efflux transporters, azole antifungal agents, *Candida albicans*

INTRODUCTION

*Candida albicans* is the major cause of oropharyngeal candidiasis (OPC), which is a frequent opportunistic infection observed in HIV-positive patients (Odds *et al.*, 1990). OPC can occur in up to 90% of these individuals during their illness (Vanden Bossche *et al.*, 1994a). To treat infections caused by *C. albicans*, only a few antifungal agents are available and among them, fluconazole, which is a compound belonging to the class of azole antifungal agents, is by far the most commonly used antifungal (Powderly, 1994). The target of azole antifungal agents in yeast is a cytochrome P-450. This enzyme is involved in the 14α-demethylation of lanosterol which is an important step in the biosynthesis of ergosterol (Vanden Bossche *et al.*, 1994b).

The repeated use of fluconazole to treat OPC episodes has led to the appearance of clinical resistance which had been often correlated with *in vitro* resistance (Odds *et al.*, 1996; Rex *et al.*, 1995; Troillet *et al.*, 1993). It was demonstrated in a recent study investigating the mechanisms of resistance to fluconazole in *C. albicans* isolates that the majority of resistant *C. albicans* isolates failed to accumulate the levels of fluconazole measured...
in susceptible isolates. This phenomenon was due to an enhanced efflux of fluconazole. Multidrug efflux transporters of two different classes were up-regulated in C. albicans azole-resistant isolates, including ABC transporters and major facilitators (MFs), and were identified as possible mediators for this effect (Sanglard et al., 1995). Each of the genes for these two transporters classes, CDR1 (Prasad et al., 1995) and CaMDR1 (Fling et al., 1991), were shown to be up-regulated in individual C. albicans isolates resistant to azole antifungal agents.

Additional mechanisms have been demonstrated to participate in azole resistance in C. albicans clinical isolates. These separate mechanisms involve alterations in the cellular target of azole antifungals (Sanglard et al., 1998) or alterations in the ergosterol biosynthetic pathway (Marichal & Vanden Bossche, 1995). However, other mechanisms of azole resistance may be still found in clinical isolates. We therefore attempted the cloning of azole resistance genes putatively involved in the resistance of clinical isolates by a functional complementation strategy. The screening of a C. albicans genomic library in a Saccharomyces cerevisiae mutant lacking the ABC transporter gene PDR5 allowed the cloning of several azole resistance genes. Previously isolated genes (CDR1 and CaMDR1) (Fling et al., 1991; Prasad et al., 1995) were found in this screening. A new ABC transporter gene, CDR2, was also isolated and was shown to be co-ordinately up-regulated with CDR1 in several C. albicans azole-resistant clinical isolates (Sanglard et al., 1997). Three additional genes were also cloned, among them ERG11 (encoding the cytochrome P450 lanosterol 14α-demethylase), FLU1 and FLU2, the latter now referred to as CAP1 and encoding a transcription factor (Alarcos et al., 1997; Sanglard et al., 1997). Here we report the characterization of FLU1 and show that it encodes a new multidrug efflux transporter with similarity to the MF transporters.

**METHODS**

**Strains.** C. albicansCAF-42 (Δura3::imm434/Δura3::imm434) and the parent CAF-21 (Δura3::imm434/URA3) are derived from the wild-type strain SC5314 and were obtained from B. Fonzi (Fonzi & Irwin, 1993). The S. cerevisiae strain YKKB-13 (MATa, ura3-52 lys2-801amber ade2-101amber trp1-Δ63 his3-a200 len2-Δ1 ADR5::TRP1) has been described by Bissinger & Kuchler (1994). The genotypes of other yeast strains are listed in Table 1. B. Fonzi (Fonzi & Irwin, 1993). The YKKB-13 was used as a host for plasmid construction and propagation. The clinical yeast strains used in this study were obtained by capillary action on GeneScreen Plus membranes (NEN). Membranes were pre-hybridized at 42 °C, with a buffer consisting of 50% formamide, 1% SDS, 4× SSC (1× SSC is 0.15 M sodium chloride, 0.015 M sodium citrate), 10% dextran sulfate and 100 μg salmon sperm DNA ml⁻¹. DNA probes were labelled with [α-32P]dATP by random priming (Feinberg & Vogelstein, 1984) and added to the hybridization solution overnight at 42 °C. Washing steps were at high stringency, identical to those recommended by the supplier (NEN). The TEF3 mRNAs were analysed using a 0.7 kb EcoRI-PstI fragment from pDC1 as described by Hube et al. (1994). Probes were stripped off in sequential hybridizations by boiling membranes for 10 min in TE buffer with 0.1% SDS.

**Northern blotting and signal quantification.** Total RNA from yeasts was extracted and electrophoresed following the method described by Sanglard et al. (1995). Transfer of RNA was performed by capillary action on GeneScreen Plus membranes (NEN). Membranes were pre-hybridized at 42 °C, with a buffer consisting of 50% formamide, 1% SDS, 4× SSC (1× SSC is 0.15 M sodium chloride, 0.015 M sodium citrate), 10% dextran sulfate and 100 μg salmon sperm DNA ml⁻¹. DNA probes were labelled with [α-32P]dATP by random priming (Feinberg & Vogelstein, 1984) and added to the hybridization solution overnight at 42 °C. Washing steps were at high stringency, identical to those recommended by the supplier (NEN). The TEF3 mRNAs were analysed using a 0.7 kb EcoRI-PstI fragment from pDC1 as described by Hube et al. (1994). Probes were stripped off in sequential hybridizations by boiling membranes for 10 min in TE buffer with 0.1% SDS.

**Southern blotting.** Genomic DNA from C. albicans strains was isolated from 5 ml cultures grown overnight in YEPD medium. Cells were digested by centrifugation and washed twice in TE. Pellets were resuspended in 5 ml PRO-Buffer (1 M sorbitol, 25 mM EDTA, 20 mM Tris/HCl, pH 7.5) and 50 μg 100T Zymolyase ml⁻¹ (Seikagaku) and 0.1% β-mercaptoethanol (Sigma) were added. The mixture was incubated at 37 °C until complete cell wall digestion occurred (up to 30 min). After centrifugation the cell pellets were slowly resuspended in 2 ml lysing solution (0.1 M EDTA, 0.8% SDS, 50 μg ml⁻¹ proteinase K, 0.1% Triton HCl, pH 7.5) and were incubated on ice for 10 min. After centrifugation (10 min at 5500 r.p.m.), supernatants were transferred into new tubes and DNA was precipitated with 5 ml ethanol. DNA pellets were gently resuspended in 0.5 ml TE containing 100 μg RNase A ml⁻¹ (Roche) and incubated at 37 °C for 15 min. DNA from each culture was then precipitated with 2-propanol, transferred into Eppendorf tubes, washed with 70% ethanol and finally resuspended in TE. DNA was digested by restriction enzymes and size-fractionated by 1% agarose gel electrophoresis. The digested DNA was vacuum-blotted on Gene Screen Plus membranes. Prehybridization and hybridization of the membrane with labelled DNA probe were performed at 42 °C in a solution containing 50% formamide, 5× SSC, 1%...
Table 1. Genotypes of yeast strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Parental strain</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAF4-2</td>
<td>CAF2-1</td>
<td>Δura3::imm434/ura3::imm434</td>
<td>Fonzi &amp; Irwin (1993)</td>
</tr>
<tr>
<td>DSY448</td>
<td>DSY447</td>
<td>Δcdr1::hisG-URA3-hisG/Δcdr1::hisG</td>
<td>Sanglard et al. (1996)</td>
</tr>
<tr>
<td>DSY465</td>
<td>DSY464</td>
<td>Δcamdr1::hisG-URA3-hisG/Δcamdr1::hisG</td>
<td>Sanglard et al. (1996)</td>
</tr>
<tr>
<td>DSY468</td>
<td>DSY467</td>
<td>Δcdr1::hisG/Δcdr1::hisG</td>
<td>Sanglard et al. (1996)</td>
</tr>
<tr>
<td>DSY653</td>
<td>DSY654</td>
<td>Δcdr2::hisG-URA3-hisG/Δcdr2::hisG</td>
<td>Sanglard et al. (1997)</td>
</tr>
<tr>
<td>DSY1</td>
<td>DSY1</td>
<td>Δura1::hisG-URA3-hisG/Δura1::hisG</td>
<td>This study</td>
</tr>
<tr>
<td>DSY2</td>
<td>DSY2</td>
<td>Δura1::hisG-URA3-hisG/Δura1::hisG</td>
<td>This study</td>
</tr>
<tr>
<td>DSY3</td>
<td>DSY3</td>
<td>Δura1::hisG-URA3-hisG/Δura1::hisG</td>
<td>This study</td>
</tr>
<tr>
<td>DSY4</td>
<td>DSY4</td>
<td>Δura1::hisG-URA3-hisG/Δura1::hisG</td>
<td>This study</td>
</tr>
<tr>
<td>DSY5</td>
<td>DSY5</td>
<td>Δura1::hisG-URA3-hisG/Δura1::hisG</td>
<td>This study</td>
</tr>
<tr>
<td>DSY6</td>
<td>DSY6</td>
<td>Δura1::hisG-URA3-hisG/Δura1::hisG</td>
<td>This study</td>
</tr>
<tr>
<td>DSY7</td>
<td>DSY7</td>
<td>Δura1::hisG-URA3-hisG/Δura1::hisG</td>
<td>This study</td>
</tr>
<tr>
<td>DSY8</td>
<td>DSY8</td>
<td>Δura1::hisG-URA3-hisG/Δura1::hisG</td>
<td>This study</td>
</tr>
<tr>
<td>DSY9</td>
<td>DSY9</td>
<td>Δura1::hisG-URA3-hisG/Δura1::hisG</td>
<td>This study</td>
</tr>
<tr>
<td>DSY10</td>
<td>DSY10</td>
<td>Δura1::hisG-URA3-hisG/Δura1::hisG</td>
<td>This study</td>
</tr>
<tr>
<td>DSY11</td>
<td>DSY11</td>
<td>Δura1::hisG-URA3-hisG/Δura1::hisG</td>
<td>This study</td>
</tr>
<tr>
<td>DSY12</td>
<td>DSY12</td>
<td>Δura1::hisG-URA3-hisG/Δura1::hisG</td>
<td>This study</td>
</tr>
<tr>
<td>DSY13</td>
<td>DSY13</td>
<td>Δura1::hisG-URA3-hisG/Δura1::hisG</td>
<td>This study</td>
</tr>
<tr>
<td>DSY14</td>
<td>DSY14</td>
<td>Δura1::hisG-URA3-hisG/Δura1::hisG</td>
<td>This study</td>
</tr>
<tr>
<td>DSY15</td>
<td>DSY15</td>
<td>Δura1::hisG-URA3-hisG/Δura1::hisG</td>
<td>This study</td>
</tr>
<tr>
<td>DSY16</td>
<td>DSY16</td>
<td>Δura1::hisG-URA3-hisG/Δura1::hisG</td>
<td>This study</td>
</tr>
<tr>
<td>DSY17</td>
<td>DSY17</td>
<td>Δura1::hisG-URA3-hisG/Δura1::hisG</td>
<td>This study</td>
</tr>
<tr>
<td>DSY18</td>
<td>DSY18</td>
<td>Δura1::hisG-URA3-hisG/Δura1::hisG</td>
<td>This study</td>
</tr>
<tr>
<td>DSY19</td>
<td>DSY19</td>
<td>Δura1::hisG-URA3-hisG/Δura1::hisG</td>
<td>This study</td>
</tr>
<tr>
<td>DSY20</td>
<td>DSY20</td>
<td>Δura1::hisG-URA3-hisG/Δura1::hisG</td>
<td>This study</td>
</tr>
</tbody>
</table>

SDS and 100 μg denatured salmon sperm DNA ml⁻¹. The probe was prepared by random priming as described above. The membrane was washed as recommended by the manufacturer and exposed at −70 °C on a Fuji X-ray film with an intensifying screen.

PCR amplifications. PCR buffers and Taq polymerase were from Boehringer Mannheim. Briefly, PCR was carried out in a Thermal Cycler 480 (Perkin Elmer) with a first cycle of denaturation for 4 min at 94 °C followed by 30 cycles of annealing at 54 °C for 2 min, elongation at 72 °C for 2 min.
and denaturation at 94 °C for 30 s. PCR was completed by a final elongation step at 72 °C for 10 min. Primers for PCR are described in the legend to Fig. 4. Yeast DNA templates for PCR were prepared from overnight cultures by mechanical breakage with glass beads as described previously (Sanglard et al., 1996).

Sequencing. Sequencing reactions were performed by standard protocols using an AutoRead kit (Pharmacia Amersham). The reactions were analysed on an ALF automated station (Pharmacia). Sequences were obtained by primer elongation using custom primers (Microsynth).

RESULTS

Isolation and sequence analysis of FLU1

Since the S. cerevisiae mutant YKKB-13 lacking the ABC transporter Pdr5p was hypersusceptible to azole antifungal agents (Sanglard et al., 1995), this phenotype was used for complementing azole hypersusceptibility with a C. albicans genomic library. As reported in a previous study, six different plasmid classes were isolated (Sanglard et al., 1995). Two classes contained the previously cloned genes CDR1 (Prasad et al., 1995) and CaMDR1 (Fling et al., 1991), and a third class contained the CDR2 gene (Sanglard et al., 1997). The fourth and fifth classes were represented by plasmids pDS257 and pDS270, which contained CAP1 (Sanglard et al., 1997) and ERG11 (D. Sanglard, unpublished data), respectively. Plasmid pDS255 belonged to the latter class and contained a gene different from those already documented. pDS255 conferred resistance to fluconazole, cycloheximide, terbinafine, sulfomethuron, cerulenin and brefeldin A in S. cerevisiae YKKB-13 (Sanglard et al., 1997).

The C. albicans DNA insert of pDS255 was subcloned into different fragments. As summarized in Fig. 1, a minimal fragment of 4200 bp in pDS255-7 was still able to confer resistance to fluconazole and cycloheximide, but not to otherazole derivatives such as itraconazole and ketoconazole. The nucleotide sequence of this fragment was determined and an uninterrupted ORF of 1833 bp, starting from the most upstream ATG codon, was detected. The gene encoding this ORF was named FLU1 (fluconazole resistance). The 5′ flanking region from this ATG codon displayed the typical structural organization of yeast promoters: an A at position −3 and a TATA box consisting of two overlapping TATA consensus sequences (TATATA and TATAAA) at position −127 (Chen & Struhl, 1988). A + T-rich regions (80% between −236 and −1, and 95% in a 28 bp tract between −109 and −82) were also observed and could serve as upstream promoter elements for the constitutive expression of FLU1, as suggested by Struhl et al. (1985). Chromosomal mapping of FLU1 revealed its presence on chromosome 7 of the C. albicans genome. FLU1 is situated on the published physical map in a location hybridizing to fosmid 11H9 (Chibana et al., 1998).

The FLU1 ORF encoded a protein with a calculated molecular mass of 67.6 kDa. A BLAST search of this ORF with available databases revealed most similarity with members of the MF superfamily (MFS). In particular, Flu1p exhibited 31.0% identity and 58.6% similarity to another MFS member, C. albicans CaMdr1p. Alignment of the two amino acid sequences showed that the N-terminal portion of Flu1p was very divergent, not only from CaMdr1p, but also from other MFS proteins (Nelissen et al., 1995). The FLU1 sequence revealed the highest similarity with YLL028w of S. cerevisiae (data not shown). The FLU1 amino acid sequence displays the structure and organization of a protein with multiple transmembrane domains. As in the case of CaMdr1p and YLL028wp, Flu1p is composed of two homologous
halves, each containing six transmembrane spans. This structural organization is a typical characteristic of MFS efflux transporters.

Expression of FLU1 in C. albicans clinical isolates

Several matched pairs of C. albicans clinical isolates were selected to address the possible expression of FLU1 in C. albicans. Six isolates were taken from a previously published study (Sanglard et al., 1995) and six additional isolates were from separate patients. Each pair contained an azole-susceptible and an azole-resistant isolate. The susceptibility to azole derivatives of these 12 isolates is given in Table 2. These clinical strains were sequential isolates from a group of HIV-positive patients and were characterized by different genotyping methods (Boerlin et al., 1996). Each of these isolates was chosen on the basis of their already known azole resistance mechanisms. Total RNA from these isolates was extracted and subjected to Northern blotting analysis with labelled probes corresponding to the multidrug efflux transporters FLU1, CaMDR1, CDR1 and CDR2. The expression of these multidrug efflux transporter genes is presented in Fig. 2. The azole-resistant isolates from three patients (II, III and V) showed up-regulation of CDR1, correlating well with a decrease in azole susceptibility. The expression of CDR2 mirrored the increased expression of CDR1 in agreement with previously published results (Sanglard et al., 1997). The azole-resistant isolates from patients I, IV and VI showed up-regulation of CaMDR1 (Sanglard et al., 1995). In contrast, FLU1 expression did not parallel azole resistance as illustrated in Fig. 2(b). FLU1 expression was higher in the azole-resistant isolate of one
pair (isolate 742); however, FLU1 expression decreased in the azole-resistant isolate of a separate pair (isolate 91). In other pairs of isolates, no variation of FLU1 expression could be measured, although the basal level of expression differed between each pair. Taken together, these results suggested that FLU1, in contrast to CDR1, CDR2 and CaMDR1, was not up-regulated in azole-resistant strains, at least in the isolates investigated here.

**Disruption of FLU1 in C. albicans**

To investigate the effect of Flu1p in *C. albicans*, the gene encoding this protein was deleted by targeted gene disruption by the method described by Fonzi & Irwin (1993). Strain CAF4-2, as well as strains with different genetic backgrounds where other multidrug transporters genes (*CDR1*, *CDR2* and *CaMDR1*) were deleted (Table 1) were utilized as recipients for transformation with a linear FLU1 fragment interrupted by the *hisG*-URA3-*hisG* cassette (see Fig. 3a). PCR was applied to screen Ura+ transformants for the correct localization of the disruption of the first *FLU1* allele. Briefly, this PCR approach consisted of using a pair of primers, one specific for *FLU1* and the other specific for the *hisG* gene contained in the disruption cassette (Fig. 3). This PCR allowed control of not only the insertion of the disruption cassette, but also its correct localization at one of the *FLU1* alleles. Fig. 4 shows the results of the PCR analysis. Each Δflu1/FLU1 heterozygote analysed after regeneration of the *ura3* genetic marker (DCY2,
**Fig. 4.** PCR monitoring of the disruption of the \textit{FLU1} gene in CAF4-2 and in five deletion mutants (DSY465, DSY448, DSY468, DSY653, and DSY654). The sequences of the primers used, \textit{P1} and \textit{P2}, are given in the legend to Fig. 3. The expected product sizes for amplification with primers \textit{P1} and \textit{P2} are 0.68 kb for a wild-type allele, 4.7 kb for the \(\Delta\text{flu1::hisG-URA3-hisG}\) allele and 1.84 kb for the disrupted allele after 5-FOA treatment (\(\Delta\text{flu1::hisG}\)). The origin of genomic DNA from each strain is indicated. The products of PCR reactions were separated on an agarose gel and were from cells after disruption of the first and second \textit{FLU1} alleles. Two bands, corresponding to the \textit{FLU1} wild-type and to the disrupted alleles, are observed in heterozygous mutants (\(\text{FLU1/}\Delta\text{flu1::hisG}\)). The 4.7 kb band, which was expected for the \(\Delta\text{flu1::hisG-URA3-hisG}\) allele was not observed, probably due to the limit of extension of the \textit{Taq} polymerase used in PCR. Molecular mass standards are indicated on the left. The PCR products from the wild-type strain SC5314 and from DNA-free reagents (--) were loaded on the right.

**Fig. 5.** Verification of \textit{FLU1} disruption by Southern analysis. Samples (2 \(\mu\)g) of genomic DNA from \textit{C. albicans} wild-type SC5314, heterozygous \(\Delta\text{flu1}\) mutants (DCY2, DCY6, DCY10, DCY13, DCY16, and DCY19) and homozygous \(\Delta\text{flu1}\) mutants (DCY3, DCY7, DCY11, DCY14, DCY17, and DCY20) were digested with \textit{SpeI}, separated by agarose gel electrophoresis and transferred onto a GeneScreen Plus membrane. The membrane was probed with a labelled \textit{FLU1} fragment as described in the legend to Fig. 2. The sizes of the \textit{SpeI} fragments hybridizing with the \textit{FLU1} probe were predicted to be 4.6 kb for the wild-type allele, 5.7 kb for the \(\Delta\text{flu1::hisG}\) allele after 5-FOA regeneration and 8.2 kb for the \(\Delta\text{flu1::hisG-URA3-hisG}\) allele. These sizes correspond approximately to those detected by the \textit{FLU1} probe, as shown on the right. Molecular mass standards are indicated on the left.

DCY6, DCY10, DCY13, DCY16 and DCY19) still contained the wild-type \textit{FLU1} allele amplified as a 0.68 kb fragment and the 1.84 kb fragment expected from the \(\Delta\text{flu1::hisG}\) disrupted allele. These \textit{C. albicans} strains, heterozygous for the first \textit{FLU1} disrupted allele, were further utilized for the disruption of the second remaining wild-type allele. No signal corresponding to the amplified wild-type \textit{FLU1} allele could be recovered from the generated homozygous mutants (DCY3, DCY7, DCY11, DCY14, DCY17 and DCY20). A complementary verification of \textit{FLU1} disruption in heterozygous and homozygous strains was monitored by Southern blotting (Fig. 5). Fragments of expected sizes were detected in this analysis and no wild-type \textit{FLU1} fragment could be observed in all homozygous deletion mutants. To verify if residual expression of \textit{FLU1} could
still be monitored in the final homozygotes, a Northern blot was performed with total RNA extracted from these strains. As shown in Fig. 6, FLU1 mRNA was detectable in the parent strain CAF4-2, as well as in multidrug transporter mutants DSY448, DSY465, DSY468, DSY653 and DSY654. However, no FLU1 mRNA could be detected in the homozygous mutants DCY3, DCY7, DCY14, DCY11, DCY17 and DCY20, thus demonstrating that disruption of FLU1 is associated with loss of expression.

**Drug susceptibility assays**

Cell viability was not altered when both FLU1 alleles were disrupted in CAF4-2 and other multidrug transporter mutants. Growth and morphological aspects of the Δflu1/Δflu1 mutants were apparently not affected. The usefulness of multidrug transporter mutants in addressing the function of multidrug transporters and assigning unrelated compounds as possible substrates of these proteins in *C. albicans* was reported previously (Sanglard *et al.*, 1996, 1997). If a defined inhibitor reduces the growth of a mutant compared to the wild-type, it suggests that this substance accumulates in the intracellular cell compartments, due to the absence of an efflux transporter specific for this substance. The difference in growth between the CAF2-1 wild-type strain and DSY448 (Δcdr1/Δcdr1) on plates containing fluconazole illustrates this effect and demonstrates that fluconazole is a substrate for Cdr1p (Fig. 7). Comparisons of growth between CAF2-1 and six different strains in which FLU1 was disrupted (DCY3, DCY7, DCY11, DCY14, DCY17 and DCY20) showed reduced growth on YEPD containing mycophenolic acid (Fig. 7) and thus indicates that mycophenolic acid is probably a substrate for Flu1p. With the exception of mycophenolic acid, where the increased susceptibility of the Δflu1/Δflu1 mutants was very noticeable, some smaller differences in growth were also seen when other compounds were added to the YEPD medium. Disruption of FLU1 in CAF4-2 resulted in increased susceptibility to the three azoles fluconazole, ketoconazole and itraconazole (Fig. 7). Susceptibility to the different agents tested was dependent on the different genetic backgrounds. As shown previously by Sanglard *et al.* (1996), the *C. albicans* Δcdr1/Δcdr1 mutant DSY448 was rendered hypersusceptible to fluconazole, ketoconazole and itraconazole, as well as to other medically relevant antifungal agents like terbinafine and amorolfine (Vanden Bossche *et al.*, 1994b), and to metabolic inhibitors such as cycloheximide (a protein biosynthesis inhibitor; Mutoh *et al.*, 1995), brefeldin A (an inhibitor of organelle assembly; Graham *et al.*, 1993) and cerulenin (an inhibitor of fatty acid biosynthesis; Morisaki *et al.*, 1993). The Δacamdr1/Δacamdr1 mutant DSY465 showed hypersusceptibility to 4-nitroquinoline-N-oxide (a mutagen; Decottignies *et al.*, 1995), but not to azole antifungal agents (Goldway *et al.*, 1995; Sanglard *et al.*, 1996). However, when the Δcdr2/Δcdr2 (DSY653) deletion mutant was exposed to the same compounds, the growth of this mutant was not affected (Sanglard *et al.*, 1997).

Some compounds failed to inhibit the growth of the Δflu1/Δflu1 mutants constructed in this study. These substances were: cycloheximide, brefeldin A, cerulenin, benomyl (an antimitic drug; Fling *et al.*, 1991), methotrexate (a dihydrofolate reductase inhibitor; Fling *et al.*, 1991), amorolfine, terbinafine and crystal violet (data not shown).

**Accumulation of [3H]fluconazole in *C. albicans***

Since the expression of FLU1 in *S. cerevisiae* mediates resistance to fluconazole, therefore assigning fluconazole as a substrate for Flu1p, we addressed the accumulation of this azole in *C. albicans* Δflu1/Δflu1 mutants. A similar experiment with the mutant DSY448 revealed a decrease in fluconazole accumulation compared to the wild-type (Sanglard *et al.*, 1996). However, a decrease in fluconazole accumulation could not be measured between Δflu1/Δflu1 mutants and the wild-type, even in the genetic background of multidrug transporter mutants (data not shown). This experiment suggests either a low efflux capacity of Flu1p for fluconazole or low expression levels of this transporter in *C. albicans*, thus making the measurement of accumulation differences between mutants and wild-type cells difficult.

**DISCUSSION**

We report here the cloning of FLU1 by functional complementation of fluconazole hypersusceptibility of an *S. cerevisiae* Δpdr5 mutant. At the outset, the purpose of this procedure was to isolate functional genes to
probe their possible involvement in theazole resistance of clinical isolates. This method of complementation permitted the isolation of at least four genes involved in the transport of fluconazole: CDR1 (Sanglard et al., 1995), CDR2 (Sanglard et al., 1996), CaMDR1 (Sanglard et al., 1995) and FLU1. Two other genes not related to the family of multidrug efflux transporters were recovered by this strategy, namely CAP1 and ERG11 (Sanglard et al., 1997). We cannot exclude that the present screening was not saturated and thus other azole resistance genes might be cloned in the future using other more drug-sensitive S. cerevisiae strains in which additional multidrug efflux transporter genes are deleted. On the other hand, using the data available from the on-going C. albicans genome sequencing project, other drug resistance genes belonging to the family of multidrug transporters may be revealed and may participate in the development of azole resistance in the clinical isolates. The rationale of functional complementation has been utilized recently (Launhardt et al., 1998)
for isolating drug resistance genes from *S. cerevisiae* with ketoconazole as a selection pressure. Among the genes recovered were ERG11, COX3 (encoding subunit I of cytochrome c oxidase), RPL27 (encoding ribosomal protein L27) and five other genes of unknown function. Thus, these additional genes, although isolated in *S. cerevisiae*, might reveal new pathways by which azole resistance could develop in *C. albicans* clinical isolates. A similar cloning approach would be practical in identifying drug resistance genes from other fungal pathogens of increasing importance, such as *Candida glabrata* or *Candida tropicalis*. We recently isolated threeazole resistance genes from *C. glabrata*, among which one, an ABC transporter gene similar to *CDR1*, was up-regulated in azole-resistant isolates (Sanglard et al., 1999).

When expressed in *S. cerevisiae*, FLU1 mediated specific resistance to fluconazole among the differentazole antifungal agents tested. The absence of cross-resistance to all threeazole derivatives in *S. cerevisiae* already suggested that the protein encoded by FLU1 could be more related to the MF CaMdr1p than to the ABC transporters Cdr1p and Cdr2p, since the expression of CaMDR1 resulted in specific resistance to fluconazole (Sanglard et al., 1995, 1997). In fact, the sequencing of *FLU1* revealed that this gene encoded a putative MF transporter. The structural organization of Flu1p was characteristic of MF efflux transporters. MFs have been grouped into three clusters (I, II and III) based on hydropathy analyses. As deduced from this analysis *FLU1* belongs to cluster I, since this transporter has 12 putative transmembrane domains (Goffeau et al., 1997).

Further studies should confirm the localization of this protein to the cytoplasmic membrane. Flu1p has the highest similarity with YLL028wp from *S. cerevisiae*. This latter transporter might be therefore functional in the efflux of fluconazole or cycloheximide. We attempted to demonstrate this function for YLL028wp; however, although the gene encoding the protein could be expressed under the control of the *ADH1* promoter, no drug resistance phenotype was obtained (data not shown).

In our study, when *FLU1* was up-regulated in the *S. cerevisiae* Δ*drd5* mutant, it increased the resistance of this yeast only to fluconazole and cycloheximide (see Fig. 1a). On the other hand, the disruption of *FLU1* in *C. albicans* did not lead to an increased susceptibility to cycloheximide, but to a small increase in susceptibility to fluconazole. Disruption of *FLU1* did not affect drug susceptibility to a great extent, except in the case of mycophenolic acid. A surprising effect resulting from the disruption of *FLU1* was that a slight increase in susceptibility to all azoles was observed compared to the wild-type, whereas expression of *FLU1* in *S. cerevisiae* could only confer resistance to fluconazole. The most likely explanation of these results could be that the absence of Flu1p in *C. albicans* affects either the expression or the activity of other transporters accepting other azoles (itraconazole or ketoconazole) as substrates. Taken together, the results of drug susceptibility assays reveal differences in two distinct approaches used to assign substrates to specific multidrug transporters. While the activity of transporters in *C. albicans* may be masked by the presence of other transporters responsible for intrinsic resistance of this yeast to various drugs and metabolic inhibitors, these activities may be better demonstrated using *S. cerevisiae* in which the *PDR5* gene was deleted. Alternatively, the differences observed with the two experimental systems may be due to differences in subcellular localization of Flu1p between both yeast species or differences in the expression of *FLU1* in both yeast species. We observed that in *C. albicans*, *FLU1* mRNA signals were comparatively very low as opposed to *CDR1* or *TEF3*. In *S. cerevisiae*, *FLU1* was expressed on the support of a YEp24-derived multicopy plasmid. Therefore, expression levels of *FLU1* may have been higher in *S. cerevisiae* and thus could influence the results of the susceptibility assays.

Some ORFs of cluster I MF transporters from different yeast species have known functions as multidrug resistance pumps. Car1p of *Schizosaccharomyces pombe* transports cycloheximide and amiloride (Jia et al., 1993), Cyhrp from *Candida maltosa* is specific for cycloheximide and hydrophobic drugs (Ben-Yaacov et al., 1994), and *CaMDR1* expression renders *S. cerevisiae* resistant to methotrexate, benomyl, cycloheximide, 4-nitroquinoline-N-oxide, sulfomethuron methyl, terbinafine, amorolfine, flufenazime, 1,10-phenantrolinide, cerulenin and benzotriazoles (Ben-Yaacov et al., 1994; Fling et al., 1991; Sanglard et al., 1997). In *S. cerevisiae*, several MF transporters have been characterized, being functional as multidrug resistant pumps: Atr1p transports aminotriazoles and 4-nitroquinolinel-N-oxide (Gompel-Klein & Brendel, 1990; Kanazawa et al., 1988), Sge1p is specific for crystal violet and ethidium bromide (Amakasu et al., 1993; Ehrenhofer-Murray et al., 1994) and Flr1p, which is the functional homologue of *CaMdr1p* in *S. cerevisiae*, can take at least fluconazole and cycloheximide (Alarco et al., 1997) or cerulenin (Oskouian & Saba, 1999) as substrate. The expression of the *S. cerevisiae* MF gene *YOR273c* conferred resistance to the antimalarial drug quinidine (Delling et al., 1998).

Even though *FLU1* encodes a transporter for fluconazole, the expression of this gene in *C. albicans* clinical isolates could not be related to their degree of azole resistance. We observed some variations in the expression of *FLU1* among the clinical isolates investigated in this study, the basis of which remains unknown. This is in contrast to *CaMDR1*, which is up-regulated from almost non-detectable levels to high levels in some azole-resistant isolates. Some indirect evidence could establish a link between the expression of *CaMDR1* and *CAP1*, a recently cloned transcription factor with a basic leucine zipper motif similar to the *S. cerevisiae* YAP1 gene. *CAP1* was shown to activate the transcription of the MF transporter *FLR1* (Alarco et al., 1997), which is functionally similar to *CaMDR1* in *C. albicans*. Interestingly, the *CaMDR1* promoter contains YAP1-like DNA recognition elements: a sequence, 5'-
TGACTCA-3', at position −737 to −731 upstream of the ATG codon, which is the optimal YAP1 binding site (Ellenberger et al., 1992; Kim & Struhl, 1995; Oliphant et al., 1989) and three other sequences that differ at position ±2 from TGACTCA. Multidrug resistance in C. albicans resulting from up-regulation of CaMDRI could therefore also be regulated by CAP1. FLU1 does not contain any conventional YAPI-like recognition element in its available promoter sequence and therefore its expression is not likely to be dependent on CAP1. These observations are in agreement with the differences in expression between CaMDRI and FLU1 in clinical isolates.

In conclusion, we have shown here that a gene cloned in S. cerevisiae by functional complementation for a drug resistance phenotype was not coupled to azole resistance in C. albicans. Development of azole resistance can be explained in most azole-resistant strains by known alterations. However, among the growing number of characterized azole-resistant isolates, some of them still acquire resistance by unknown mechanisms. By using a prospective functional screening of C. albicans drug resistance genes in suitable yeast genetic backgrounds, these additional remaining azole resistance mechanisms may be revealed in the future.

ACKNOWLEDGEMENTS

This work was supported by a grant No. 3100-04716 from the Swiss National Foundation to D.S. Our thanks to F. Ischer for excellent technical assistance.

REFERENCES


Received 31 March 2000; revised 30 June 2000; accepted 20 July 2000.